Structures of DNA and RNA polymerases and their interactions with nucleic acid substrates

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DNA and RNA polymerases are enzymes that are primarily responsible for copying genetic material in all living systems. The four polymerases whose structures have been determined by X-ray crystallographic methods have significant similarities at the polymerase active site that are indicative of common requirements for polynucleotide synthesis. Structural studies of complexes of the Klenow fragment of *Escherichia coli* DNA polymerase I, HIV type 1 reverse transcriptase, and rat DNA polymerase β with DNA are leading to generalized models for catalysis.

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Introduction

During the past several years there has been significant growth in our knowledge of the structures of nucleotide polymerases and their interactions with nucleic acid substrates. Although a variety of proteins are involved in the copying of genetic information, polymerases that catalyze the addition of nucleotides to a polynucleotide chain are at the heart of the process. We now know the details of the three-dimensional structures of four nucleotide polymerases: the Klenow fragment of *E. coli* DNA polymerase I, HIV-1 reverse transcriptase (RT), bacteriophage T7 RNA polymerase, and rat DNA polymerase β (pol β). In addition to the polymerase domains, Klenow fragment contains a 3′→5′ exonuclease and HIV-1 RT contains an RNase H. There is extensive similarity in the overall architecture among these four polymerases, apparently dictated by structural requirements for polynucleotide synthesis (Fig. 1). All of these polymerases contain a groove into which the nucleic acid binds. The general shape of the polymerase domains can be likened to a right hand and the subdomains of the various polymerases are referred to, by analogy, as fingers, palm, and thumb. The active sites, which contain catalytically essential acidic amino acids, are located in the palm subdomain of each of the polymerases. These acidic residues bind the divalent metal cations that are required for polymerization. This article will focus on the structures of Klenow fragment, HIV-1 RT, and pol β in complexes with nucleic acid. Because this is a brief review and the field has involved contributions from a large number of people, we have attempted to be quite selective in our citations.

Klenow fragment of *E. coli* DNA polymerase I

The Klenow fragment is a C-terminal proteolytic product (68 kDa) of *E. coli* DNA polymerase I that has DNA polymerase and 3′→5′ exonuclease activities [1]. The crystal structure, which was solved ten years ago, showed that the Klenow fragment is composed of two separate domains: the polymerase domain at the C terminus and the 3′→5′ exonuclease domain at the N terminus [2]. Several crystal structures of Klenow fragment in complex with nucleic acids have also been reported [3,4,5••,6•]. Beese *et al.* [5••] reported the structure of Klenow fragment with a covalent adduct of an epoxy-modified template–primer that may correspond to an 'editing complex' (Figs 1e, 2). In this structure, the DNA template–primer was bound in the groove between the thumb subdomain of the polymerase and the 3′→5′ exonuclease domain, rather than in the DNA-binding cleft of the polymerase itself (Fig. 2). A portion of the duplex region of the DNA interacted with α-helices of the 'thumb' subdomain of the structure. The thumb moves toward the 3′→5′ exonuclease domain when DNA is bound in this fashion, thus producing a second DNA-binding groove. In this structure the primer strand of the nucleic acid was not bound at the polymerase active site. Beese *et al.* [5••] suggested that, during poly-

Abbreviations

d—deoxy; **dd**—dideoxy; **NTP**–nucleoside triphosphate; **pol β**—rat DNA polymerase β; **RT**—reverse transcriptase; **19/18 dsDNA**—19-mer/18-mer double-stranded DNA template–primer.

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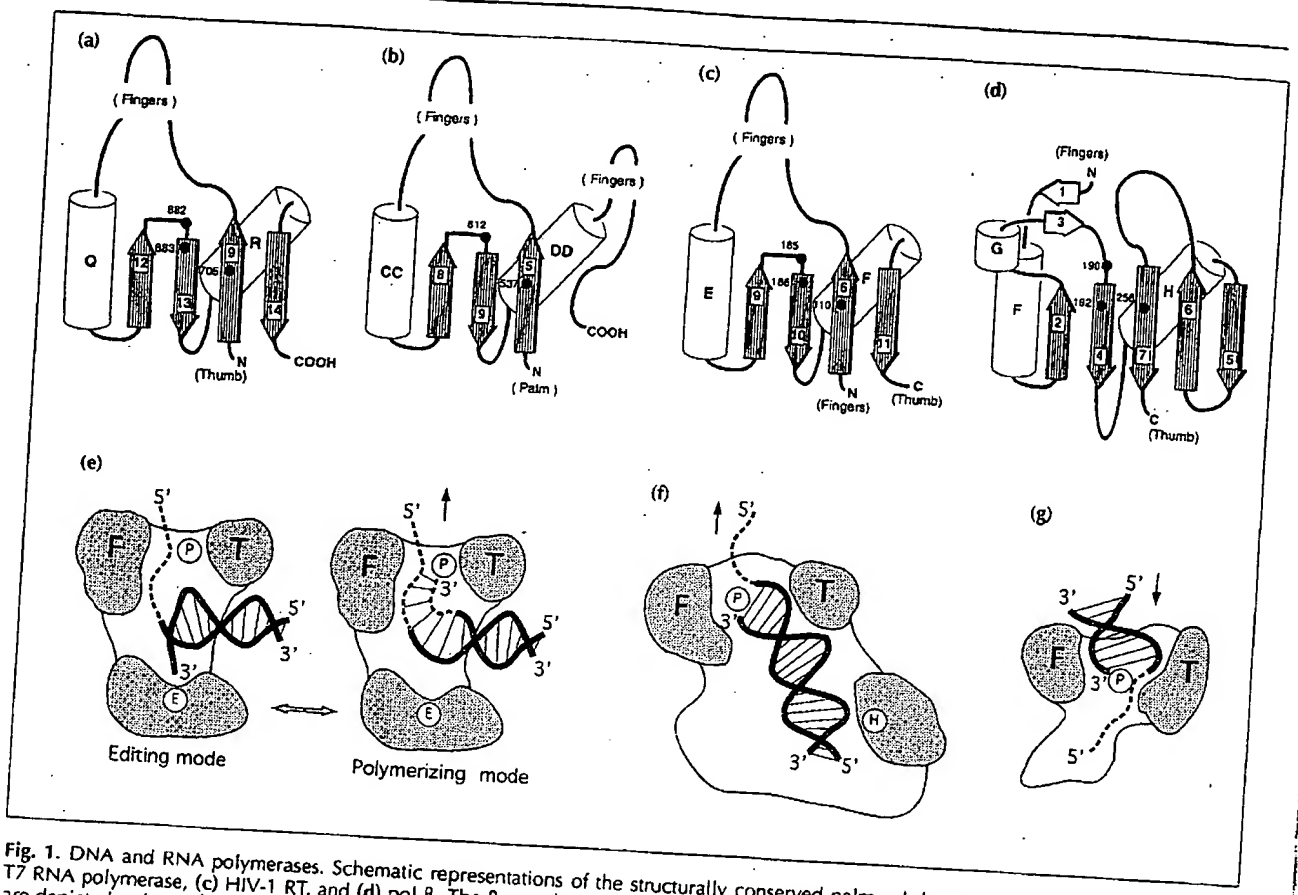

Fig. 1. DNA and RNA polymerases. Schematic representations of the structurally conserved palm subdomains in (a) Klenow fragment, (b) T7 RNA polymerase, (c) HIV-1 RT, and (d) pol β. The β-strands in the central β-sheet are depicted as numbered striped arrows, and helices are depicted as lettered cylinders. For Klenow fragment, T7 RNA polymerase, and HIV-1 RT, the interrupted loops at the top of the diagrams represent departures of the main chain from the palm into the fingers subdomain. The positions of the catalytically important carboxylate residues are shown as black dots accompanied by residue numbers. The labeling of secondary structural elements of the pol β subdomain follows that defined by Davies *et al.* [54••]. Helices αF–αH and β-strands β2, β4–β7 shown here were named αJ–αL and β1–β5, respectively, by Sawaya *et al.* [55••]. Note the differences in the topology of the palm subdomain between pol β and the other three polymerases. Complexes of DNA polymerases with DNA. (e) Klenow fragment, (f) T7 RNA polymerase, and (g) pol β.The schematic diagrams, drawn approximately to scale, summarize the pertinent points relating to the binding modes of DNA polymerases and nucleic acid substrates as derived from the available crystal structures. Fingers and thumb subdomains are labeled F and T, respectively. In pol β, the designation of fingers and thumb is based on the analogous position of such subdomains in other polymerases relative to conserved features of the palm. Depiction of modeled editing and polymerizing modes in the Klenow fragment was adapted from Fig. 6 of Steitz [7•]. DNA strands observed in the crystal structures are drawn as solid black lines. Modeled extensions of DNA strands which were not seen in the crystal structures are indicated by dashed lines. Locations of the polymerase, 3′→5′ exonuclease, and RNase H catalytic sites are shown as circled P, E, and H, respectively. The apparent direction of DNA synthesis relative to the conserved features of the palm subdomain for each polymerase is indicated by a vertical arrow.

merization, the DNA template–primer may be bent by approximately 80° to enter the DNA-binding cleft that contains the polymerase catalytic site from the direction of the 3′→5′ exonuclease domain. A subsequent publication reported the structures of Klenow fragment with bound deoxynucleoside triphosphates; however, as these structures did not include template–primers, it is not clear if the deoxynucleoside triphosphates were bound in a position relevant for polymerization [6•]. These results have been extensively reviewed by Steitz and co-workers [7•,8,9•].

HIV-1 reverse transcriptase

In 1992, Kohlstaedt *et al.* [10••] described the structure of HIV-1 RT in a complex with the non-nucleoside inhibitor nevirapine at 3.5 Å resolution; the structure has subsequently been refined to 2.9 Å resolution [11•]. Jacobo-Molina *et al.* [12••] published a 3.0 Å resolution structure of HIV-1 RT in complex with a 19-mer/18-mer double-stranded DNA template–primer (19/18 ds-DNA) and an antibody Fab fragment. The structure of HIV-1 RT in the absence of a bound inhibitor or template–primer has been described at low resolution [13•] and will be reported soon at higher resolution ([14••]; R Raag *et al.*, unpublished data) as will the structure of a complex with an α-anilino phenylacetamide (α-APA) non-nucleoside inhibitor (J Ding *et al.*, unpublished data). Unge *et al.* [15•] have described a 2.2 Å resolution structure of a truncated version of HIV-1 RT corresponding to the fingers and palm subdomains, and (MM Georgiadis *et al.*, personal communication) have solved the structure of the analogous

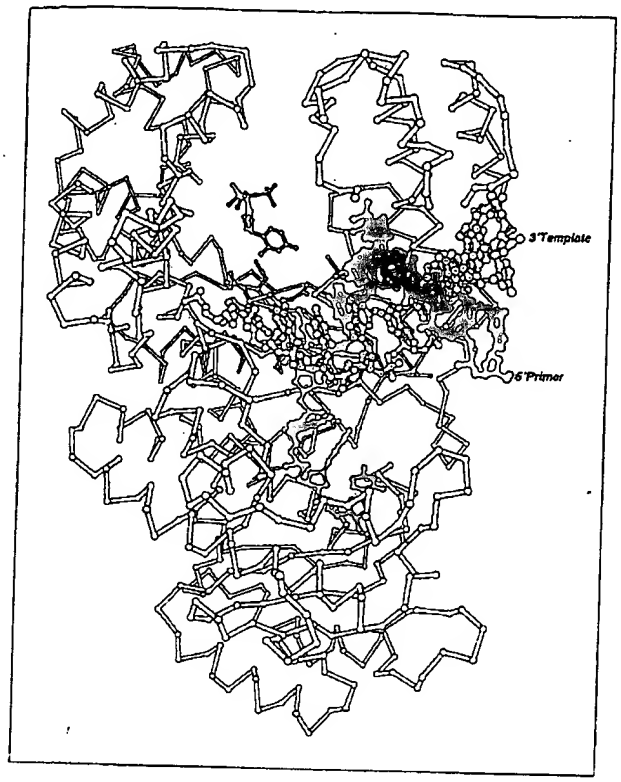

Fig. 2. The structure of Klenow fragment in what may correspond to an 'editing' complex with a template–primer DNA [5••]. The protein is represented as an α-carbon backbone model with the polymerase domain in open bond and the 3′→5′ exonuclease lightly shaded. The three aspartic acid residues at the polymerase active site are shown with side chains. The bound DNA is shown as a ball and stick model. A dCTP substrate is shown in a position which is derived from the structure of the Klenow fragment–dCTP binary complex [6•] (reprinted with permission from [8]).

portion of Moloney murine leukemia virus RT at 1.8 Å resolution. Stammers *et al.* [16] have described a crystal form of HIV-1 RT in complex with non-nucleoside inhibitors. The crystals diffract X-rays to 2.2 Å resolution and several structures resulting from this work should be reported soon.

The overall structure of the HIV-1 RT–DNA complex is shown in Fig. 3. The HIV-1 RT p66/p51 heterodimer is a highly asymmetric structure in which the polymerase domains of the p66 and p51 subunits are arranged quite differently, in spite of being identical in amino acid sequence. This asymmetry is in accord with biochemical and genetic data that had suggested that only the p66 subunit in the p66/p51 heterodimer had polymerase activity [17–19]. The polymerase active site is located in the p66 palm subdomain. The fingers, palm, and thumb subdomains all have extensive interactions with nucleic acid. Amino acid sequence motifs that had previously been identified as being highly conserved in all RTs [20,21] and most RNA-dependent polymerases [22–24], were found to cluster in the vicinity of the polymerase active site. Poch *et al.* [23] and Delarue *et al.* [24] identified five conserved sequence motifs present in all RNA-dependent polymerases A–E. Motifs A and C contain the residues that comprise the polymerase active site and the site at which dNTPs are bound before incorporation (Fig. 4). The three aspartic acids in the HIV-1 RT polymerase active site and corresponding residues in the active sites of Klenow fragment, T7 RNA polymerase, and DNA pol β are structurally equivalent. Structural elements of the palm and fingers subdomains of HIV-1 RT were found to form a clamp-like structure that holds the template–primer in a precise orientation relative to the polymerase active site. These elements were named 'primer grip' and 'template grip' and include sequences from conserved motifs E and B, respectively [12••]. Most of the contacts between enzyme and the template–primer involve the sugar-phosphate backbone of the DNA and are therefore not sequence specific. Results of modeling experiments taken together with biochemical data suggested that the fingers subdomain contacts the single-stranded template and may function to 'thread' the template strand into the polymerase active site. Helices αH and αI of the thumb subdomain of p66 interact with the primer and template strands. Helix αI is partially inserted into the minor groove of the dsDNA in the vicinity of a bend in the template–primer (discussed below). There appears to be considerable flexibility of the p66 thumb subdomain. If nothing is bound to HIV-1 RT, the thumb lies near the fingers ([14••]; R Raag *et al.*, unpublished data), whereas in the presence of either bound dsDNA [12••] or a non-nucleoside inhibitor ([10••]; J Ding *et al.*; unpublished data), the thumb moves away from the fingers by approximately 30 Å and is in an upright position.

The dsDNA bound to HIV-1 RT has an unusual and unexpected geometry. The majority of the 18 base pair duplex region is B-form, but in the vicinity of the polymerase active site, there are about 6–7 A-form base pairs. Retroviral RTs must use dsRNA and RNA : DNA template–primers during replication of the retroviral genome [25]. RNA : RNA duplexes are A-form and RNA : DNA duplexes are H-form [26•]. As both of these structures are more similar to A-form than to B-form DNA, the geometry of the polymerase active site, which must be able to accommodate all three kinds of duplexes, may induce A-form geometry in a dsDNA template–primer. At the junction between the A-form and B-form regions of the template–primer, there is a 40–45° bend that widens the minor groove. A similar structure was observed in NMR studies of a synthetic oligonucleotide RNA-DNA : DNA duplex that resembles an Okazaki fragment [27]. In this structure the RNA : DNA duplex assumes the H-form and the DNA : DNA duplex is B-form. The junction between these two regions is bent, with a widened minor groove. Ding *et al.* [28] analyzed the surface areas of contact in the HIV-1 RT–DNA complex and found that about 2100 Å^2 of a total of 7000 Å^2 of solvent-exposed surface of the template–primer is buried by interactions with the HIV-1 RT enzyme. It has been suggested that as most of the interactions are between the polymerase

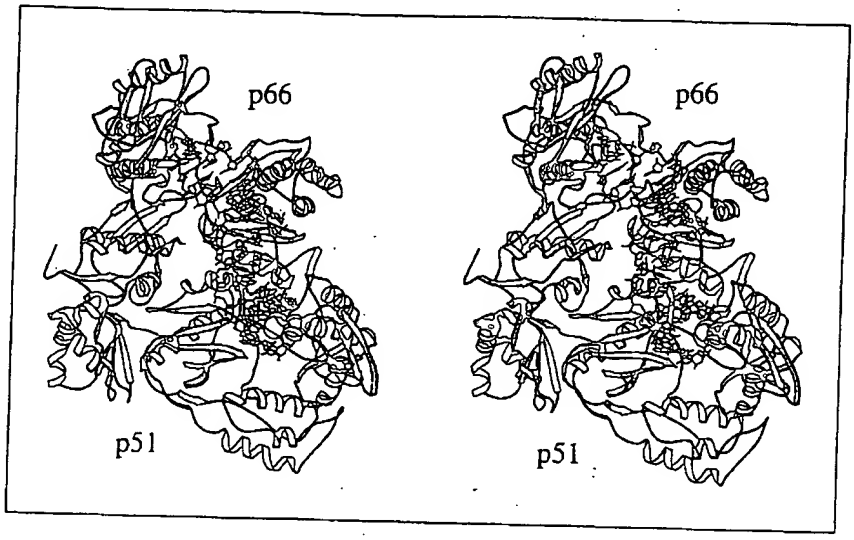

Fig. 3. A stereo folding diagram [79] of the HIV-1 RT–DNA complex, showing the DNA-binding cleft and the orientation of the bound DNA [12••]. The p66 and p51 subunits are depicted in ribbon representations and the template–primer DNA is represented by a ball and stick model.

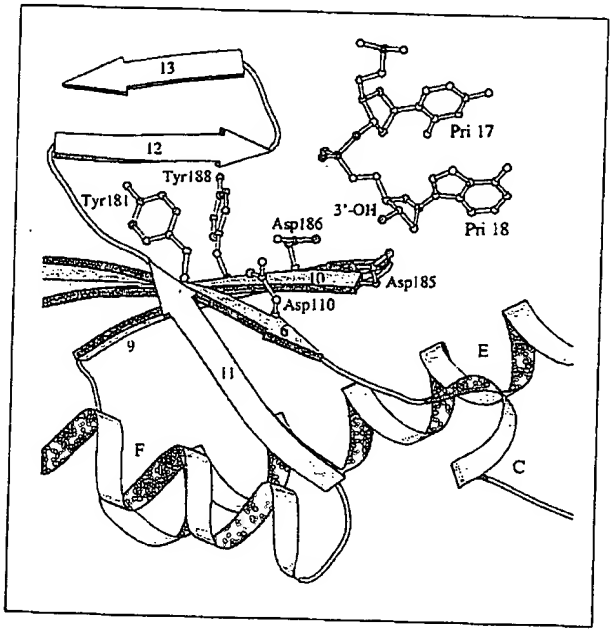

Fig. 4. Structure of the polymerase active site of HIV-1 RT showing the relative positions of the three catalytically essential aspartic acid residues Asp110, Asp185, and Asp186, and the 3′-terminal nucleotides of the primer strand of the bound DNA. Tyr181 and Tyr188, which are also shown with side chains, form part of the hydrophobic pocket to which non-nucleoside inhibitors of HIV-1 RT bind (reprinted with permission from [12••]).

domain of HIV-1 RT and the A-form region of the ds-DNA template–primer, dehydration via protein competition with water molecules may partly induce A-form geometry [27].

The RNase H domain of HIV-1 RT is located at the C-terminal end of the p66 subunit. The first RNase H structure to be determined was that of the *E. coli* enzyme, which was described by two independent groups [29,30]. The structure of the isolated HIV-1 RNaseH domain has also been determined by two groups [31,32].

There is a remarkable three-dimensional similarity between the *E. coli* enzyme and the RNase H domain of HIV-1 RT. In the HIV-1 RT p66/p51 heterodimer, there are significant contacts between RNaseH and the connection subdomains of both p51 and p66, and with the p51 thumb subdomain [10••,28]. In the HIV-1 RT–DNA structure, the polymerase and RNase H active sites are separated by approximately 17–18 nucleotides [12••]. Because the predominant contacts with the dsDNA template–primer occur in the vicinity of the polymerase active site, the precise placement of the template strand relative to the RNase H active site may be regulated by the composition and sequence of the template–primer nucleic acid. In particular, the structure of an RNA : DNA hybrid may help to place the RNA template strand in a position so that it can be cleaved by RNase H [12••,33].

An important area of research has involved the development of resistance to HIV-1 RT inhibitors that are used in the treatment of AIDS (reviewed by Tantillo *et al.* [34•]). An interesting twist on the importance of protein–nucleic acid interactions in HIV-1 RT has been proposed by Boyer *et al.* [35•], who suggested, on the basis of both structural and biochemical studies, that a number of mutations that caused HIV-1 RT to have reduced sensitivity to nucleoside analogs may exert their effect via interactions with nucleic acid substrates. Most of the mutations that cause resistance to nucleoside analogs do not occur at the site of dNTP binding, but instead are located at positions that contact the nucleic acid template–primer [10••,34•–36•]. In their model, Boyer *et al.* [35•] suggested that the mutations would affect discrimination between normal nucleoside triphosphate substrates and the analogs by changing either the position of the template–primer relative to the enzyme or the conformation of the nucleic acid.

In addition to the mechanisms of drug resistance, the structure of HIV-1 RT, combined with biochemical information, has been used to postulate the mechanism of

DNA polymerization [7°,9°,37°]. The general pathway for nucleotide addition involves the sequential binding of RT with DNA and then with dNTP; this is followed by a nucleophilic attack step leading to the formation of a phosphodiester bond and the subsequent release of the pyrophosphate moiety [38, 39, 40°]. An examination of how HIV-1 RT may respond structurally during each step has been conducted by Arnold and co-workers [37°].

Binding of template–primer may first occur with the single-stranded region of the template, followed by a movement of approximately 30 Å of the tip of the p66 thumb to accommodate duplex binding at the polymerase active site ([14••,37°]; R Raag *et al.*, unpublished data). This conformational change permits numerous interactions to occur between dsDNA and residues of helices αH and αI of the p66 thumb subdomain and results in the conformation of HIV-1 RT described in [12••]. The second step in polymerization is binding of the next nucleotide to be added to the 3′ end of the primer. Molecular modeling of an incoming dNTP based in part on the position of the mercury atom in the ternary complex between HIV-1 RT, DNA and HgUTP suggests that portions of secondary structural elements αC–β6, αE, β9–β10, and β11b set the topology of the dNTP-binding site [34°,37°]. A conformational change has been proposed to occur upon dNTP binding; this structural change could be crucial for polymerase fidelity [40°] and could involve rearrangements of portions of both protein and nucleic acid [37°]. Subsequent to the conformational change, polymerization is postulated to occur by a general two metal ion phosphoryl-transfer mechanism thought to be common to HIV-1 RT [7°,9°,37°], Klenow fragment [7°,9°], and rat DNA polymerase β [41••]. Arnold and co-workers [37°] propose that one divalent cation coordinates Asp110 and Asp186 and the β- and γ-phosphates, and that the second divalent cation chelates Asp185 and the α-phosphate before the nucleophilic attack step.

T7 RNA polymerase

RNA polymerase from bacteriophage T7 is the only RNA polymerase whose three-dimensional structure has been determined [42••]. T7 RNA polymerase is a 99 kDa monomer that carries out promoter-specific RNA transcription both *in vivo* and *in vitro*. The protein is folded into two domains. The N-terminal domain of T7 RNA polymerase is located in a position that is similar to that of the 3′→5′ exonuclease domain of Klenow fragment, but does not appear to be functionally equivalent to any of the domains of either Klenow fragment or HIV-1 RT. This domain appears to bind nascent RNA and may also be involved in binding an NTP in the absence of a primer. Both of these functions are specific to RNA polymerases [43,44].

Comparison of the structures of T7 RNA polymerase, the Klenow fragment, and HIV-1 RT emphasized the importance of conserved sequence motifs and the conservation of the geometry of the catalytically relevant acidic residues at the polymerase active site [45]. The C-terminal portion of T7 RNA polymerase, which comprises the polymerase domain, is remarkably similar in its secondary structure to the polymerase domain of the Klenow fragment. Both Klenow fragment and T7 RNA polymerase have fingers subdomains that are predominantly α-helical, as opposed to HIV-1 RT, in which the fingers subdomain has an α/β fold. The fingers, palm, and thumb subdomains form a large, deep cleft which model building suggests could accommodate a 20 base pair duplex DNA [42••]. The putative catalytic site of T7 RNA polymerase lies at the bottom of the DNA-binding cleft and contains two aspartic acid residues (Asp537 and Asp812), which are highly conserved in all polymerases, and Lys631, Tyr639 and Gly640, which are invariant in all DNA-directed polymerases but not in the RNA-directed polymerases.

An important difference between a DNA polymerase and DNA-dependent RNA polymerases is that the former make relatively long double-stranded products and the latter make duplex products only transiently in the process of making single-stranded mRNA transcripts. Biochemical analyses by Chamberlain and Goldfarb and their co-workers suggests that *E. coli* RNA polymerase may have as little as two to three base pairs of DNA : RNA duplex [46–48; 49°,50], considerably less than the approximately 12 base pair RNA : DNA duplex intermediates that have been suggested by von Hippel and Hearst and their co-workers [51–53]. It would be interesting to see how the three-dimensional structures of these complexes relate to the corresponding structures of DNA polymerases.

Rat DNA polymerase β

During the past year several structures of rat DNA polymerase β (pol β) have been determined independently by two groups. The structures represent binary complexes of pol β with dNTPs in the presence of divalent metal ions [54••,55••], as well as ternary complexes with DNA template–primer and ddCTP [41••]. Mammalian pol β is composed of two distinct domains. The overall structure of the C-terminal 31 kDa catalytic domain contains a U-shaped cleft that is characteristic of all known polymerases. Although pol β has subdomains that are analogous to the fingers and thumb of other polymerases, there is no structural homology. The decision to designate one of these subdomains as the fingers and the other the thumb in pol β was made strictly on the basis of their positions relative to the conserved features of the palm subdomains. The N-terminal 8 kDa template-binding domain, which has no counterpart in the other structurally characterized polymerases, is connected to

the fingers subdomain by a flexible protease-sensitive linker [56]. Although the active-site region of pol β was found to contain a set of structural elements that were similar to what had been found in the three other polymerase structures, Klenow fragment, HIV-1 RT, and T7 RNA polymerase are more closely related to each other than to pol β. In fact, a strong case can be made, on the basis of the different topological connectivity of the catalytic site region, that pol β represents a separate structural class of polymerases [54••,55••]. The topology of the β-sheet that carries the three catalytically important aspartic acid residues differs from that of the β-sheet at the catalytic site of the three other polymerases (Figure 1a–d), in that strand β7 of pol β (containing Asp256) is parallel to β4 (containing Asp190 and Asp192). In the other three polymerases this sheet is antiparallel. The palm subdomains of all four polymerases share a common set of secondary structural elements consisting of two α-helices and two antiparallel β-strands that form an αββα fold (corresponding to αE-β9-β10-αF in HIV-1 RT; see Fig. 1c), which is commonly found in many other protein structures. In Klenow fragment, HIV-1 RT, and T7 RNA polymerase the two β-strands of this structural unit form a tight β-turn, whereas in pol β the two corresponding β-strands are connected by a 12-residue loop, which contains an additional one-turn helix (αG) and a short β-strand. Despite the fact that the catalytic residues Asp190 and Asp192 of pol β, located at the C-terminal end of this loop, are separated by one residue, their carboxylate groups assume a very similar spatial arrangement for coordination of metal cations as do the analogous carboxylates in Klenow fragment and HIV-1 RT, which are located adjacent to one another at the tip of a tight β-turn.

Although there is significant structural similarity between the polymerase active site of pol β and the other three polymerases, it is not clear whether this similarity is the result of divergent or convergent evolution. The conserved features of the palm subdomains and the geometry of the catalytic sites may be an indication of common function rather than common origin.

Pelletier *et al.* [41••] described the structure of two ternary complexes of rat pol β with a dideoxy-terminated template–primer and ddCTP (at 2.9 Å and 3.6 Å resolution; Fig. 5). Both structures show a ddCTP bound to the active site. The catalytic triad of aspartic acid residues interact with the ddCTP via two chelated Mg^{2+} ions (Fig. 6). The duplex region of the nucleic acid and the first 5′-template nucleotide are well ordered, although the remaining three nucleotides of the single-stranded template extension were not visible in the electron density map. The majority of the contacts of pol β with the template–primer (which were relatively sparse) involved hydrogen bonds between protein main-chain amide nitrogens and phosphate oxygens of the first three base pairs of duplex DNA downstream of the active site. Thus the template–primer binding is largely independent of the DNA sequence, which is similar to what had also been found in the HIV-1 RT–DNA complex [12••]. Like the HIV-1 RT–DNA complex, the DNA duplex was found to have A-form geometry in the vicinity of the active site in the rat DNA pol β complexes with DNA. Unlike the HIV-1 RT–DNA complex, the pol β complexes do not show the duplex binding in a groove formed by the fingers, palm, and thumb subdomains, but instead the duplex DNA binds to the β-sheet of the palm subdomain that contains the active site, and there are few protein–DNA contacts beyond the first three base pairs of the duplex. The flat face of the base pair made by ddCTP and the 5′-template nucleotide interacts with the side of helix N of the thumb subdomain (Fig. 6).

On the basis of the structures of the ternary complexes with template–primer and ddCTP, Pelletier *et al.* [41••] proposed an overall polymerization mechanism that is summarized briefly here. Numerous features of the transition state in this proposed mechanism are analogous to those in the two-metal ion mechanism

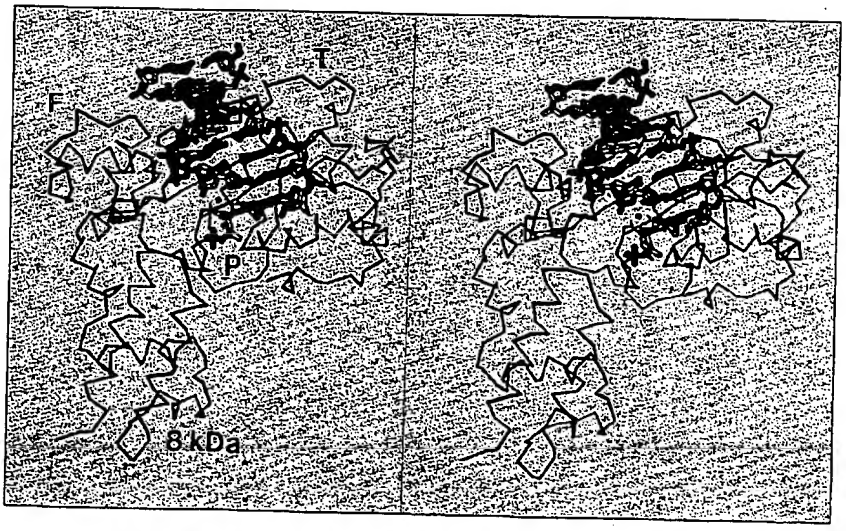

Fig. 5. Stereo diagram of rat DNA polymerase β (α-carbon backbone) in a complex with DNA and ddCTP (thick line) from the structure determined in space group $P6_1$ [49•]. Positions of the fingers (F), thumb (T), and palm (P) subdomains as well as that of the N-terminal 8 kDa domain are indicated.

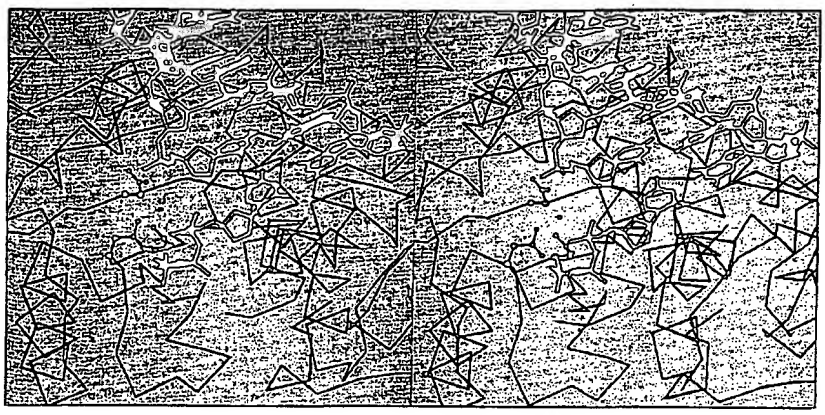

Fig. 6. Stereoview of the pol β catalytic site showing the interaction of ddCTP with the surrounding elements in the structure of Pelletier *et al.* [49••]. The DNA template–primer and ddCTP are drawn in thick line, the α-carbon backbone of pol β is in thin line. The position of a Mg^{2+} ion is indicated by a black dot between the three catalytically important apartic acid residues Asp190, Asp192, and Asp256, shown in a ball and stick model.

proposed by Beese and Steitz [4] for DNA cleavage by the 3′→5′ exonuclease of Klenow fragment and by Steitz [7•] for DNA polymerization. Binding and positioning of the ddNTP is accomplished by hydrogen bonding to the complementary template base and by hydrogen bonds and van der Waals interactions with Asn279 and Asp276. Six hydrogen bonds are formed between protein atoms and ddCTP phosphate oxygens. Two Mg^{2+} ions are chelated by Asp190, Asp192, and Asp256 and by the phosphate oxygens of the triphosphate. The role of one Mg^{2+} ion was suggested to include activation of the 3′-OH of the primer terminus as an alkoxide nucleophile, with Asp256 acting as a general base for abstraction of the hydroxyl proton. It was also suggested that Mg^{2+} would stabilize the pentacoordinated α-phosphate intermediate in the transition state. A second Mg^{2+} ion acts primarily to chelate the β- and γ-phosphates of the ddCTP and may remain associated with the pyrophosphate leaving group after the covalent attachment of the nucleotide to the growing DNA chain.

The orientation of template–primer in polymerases

The observation that the structures of the palm regions of Klenow fragment, HIV-1 RT, and rat pol β, (including the three conserved catalytically essential carboxylates) can be superimposed, suggests that there are common structural requirements for DNA synthesis. It was quite unexpected to find that the template–primer in the pol β complex has an orientation opposite to that seen in the HIV-1 RT–DNA complex and to the current models for Klenow fragment binding to DNA. Pelletier *et al.* [41••] suggested that the ternary complex between pol β, DNA and ddCTP represents a physiologically relevant form and that the detailed mechanistic implications of this structure would be applicable to all polymerases. To be consistent with the idea that all polymerases share a common catalytic mechanism, these authors proposed that the orientations of template–primer in other polymerase–DNA models were incorrect. They suggested that RT, as a special case, could accommodate the template–primer in two possible orientations, in which the one seen in the structure of the HIV-1 RT–DNA complex would be used with RNA templates, and the orientation seen in the pol β–DNA complex would be used with DNA templates. The latter possibility would require interchangeable roles for the catalytic residues in RT.

HIV-1 RT has the same orientation when it copies RNA and DNA templates

There is a wealth of structural and biochemical information that makes it clear that HIV-1 RT binds all of its nucleic acid template–primer substrates (RNA : RNA, RNA : DNA, and DNA : DNA) in the same orientation. Among the strongest arguments that RTs are oriented in the same direction on RNA and DNA templates is that the vast majority of point mutations have similar effects on both RNA-dependent and DNA-dependent polymerization [35•, 57, 58]. Pelletier *et al.* [41••] specifically proposed that in polymerization using a DNA template the tyrosine (Tyr183 in HIV-1 RT) of the highly conserved YMDD motif (one-letter amino acid code used) of RTs might functionally replace the catalytically essential Asp110; however, a Tyr183→Phe mutant HIV-1 RT polymerizes from both templates at roughly the same efficiency (approximately one-third that of the wild-type enzyme), whereas HIV-1 RT with an Asp110→Glu mutation was unable to copy either RNA or DNA templates efficiently [57]. The Tyr183→Phe HIV-1 RT mutant lacks the hydroxyl group that would be essential for DNA-dependent DNA polymerase activity in the model proposed by Pelletier *et al.* [41••]. Similar arguments can be made for a large number of other mutations.

Other independent evidence argues for the equivalent orientations of template–primer binding to HIV-1 RT for both RNA and DNA templates. The orientation of

RT on RNA templates can be deduced from experiments that measure the relative positions of the polymerase and RNase H active sites. If the reaction conditions are chosen so that the polymerase and RNase H reactions are coupled, it can be seen that the HIV-1 RNase H active site 'trails' the polymerase active site by some 14–18 base pairs [59–61]. This is the logical arrangement, as the RNase H cleavage of the RNA template strand can occur on newly formed RNA:DNA duplex products after a DNA complement of the RNA has been made.

The strongest argument for the orientation of HIV-1 RT on DNA templates comes from the structure of the HIV-1 RT:DNA complex [12••]. The 18 base pair duplex region of the 19/18 dsDNA occupies the binding cleft formed by the fingers, palm, and thumb subdomains of HIV-1 RT and spans the region between the polymerase and RNase H active sites. Even a cursory examination of the structure suggests that this is the logical location of the nucleic acid substrate, as it provides the enzyme with the opportunity to grasp and hold the relatively rigid duplex region of the template–primer and, in so doing, position the 3′-OH of the primer terminus at the polymerase active site. More evidence that the 3′-OH is in the correct orientation relative to the polymerase active site comes from observations that a mercurated dNTP can be bound to the polymerase active site in this complex [12••,62]. Recent footprinting experiments with DNase I indicate that about 20–22 base pairs of DNA are protected by HIV-1 RT [63], in good agreement with what one would predict from the separation of polymerase and RNase H active sites. If the DNA were in the orientation suggested by Pelletier *et al.* [41••], then only a small number of base pairs (approximately six to eight) would be protected from DNase I cleavage by HIV-1 RT. Furthermore, enzymatic footprinting of complexes between DNA and HIV-1 RT mutants containing small deletions in the RNase H subdomain gave slightly smaller duplex DNA footprints than did wild-type enzyme. These experiments indicate that the RNase H subdomain is adjacent to the duplex region of the DNA (SFJ Le Grice, personal communication). Footprinting of the HIV-1 RT–DNA complexes with S1 nuclease suggests that a single-stranded region of about seven nucleotides is shielded by HIV-1 RT, consistent with predicted interactions of the template with the p66 fingers subdomain [63]. Not surprisingly, the results of both chemical [64] and enzymatic footprinting experiments are in accord with the structure of the HIV-1 RT–DNA complex.

Can the structures of the pol β complexes with DNA be extrapolated to other polymerases?

Unlike the HIV-1 RT–DNA complex, the complex of pol β with DNA is more difficult to correlate with the available biochemical data. Because the interpretation of the ternary complex between pol β, DNA and dd-CTP as described by Pelletier *et al.* [41••] has potentially far-reaching consequences for the mechanism of DNA polymerization, the biological and catalytic relevance of this complex should be examined carefully. The following five points should be considered before the binding mode of pol β with short oligonucleotides is accepted as the paradigm for polymerase–DNA interactions.

First, although biochemical data suggest that the positively charged N-terminal domain of pol β plays a major role in binding DNA substrates [56,65], this 8 kDa domain does not interact with the template–primer in the complexes between pol β and the template–primer. Surprisingly, the position of the highly flexible N-terminal domain does not appear to be relevant for substrate binding in either of the two crystal forms. Pelletier *et al.* [41••] suggest that an extended single strand of the template could make contacts with the N-terminal domain.

Second, gapped duplex DNA, with double-stranded regions both upstream and downstream of the site of nucleotide incorporation, is the natural substrate for pol β [66]. Results obtained by Wilson and colleagues [65,67] suggest that pol β is able to completely fill short single-stranded gaps via a processive mechanism that strictly requires a 5′-phosphate at the downstream boundary of the gap, a feature not present in the complex with short DNA template–primers. Given the approximate twofold symmetry of a gapped DNA duplex, the predominant contacts of main-chain amide nitrogens with phosphate oxygens of the template–primer in the pol β–DNA complex could potentially be satisfied by binding to the upstream duplex region if a gapped DNA duplex substrate were bound to pol β in an orientation opposite to that observed with the short oligonucleotides.

Third, the axis of the double-helical region of the DNA substrate in the crystal points directly to a wall formed by helices αM and αN of the thumb subdomain of pol β (these helices are called αI and αJ in the nomenclature of Davies *et al.* [54••]). A longer, more biologically relevant substrate would have to bind quite differently to fit the DNA-binding groove or, alternatively, a sharp bend of the template strand would be necessary to preserve the binding mode of DNA as seen in the pol β complexes containing short oligonucleotides. It would be particularly difficult for the observed pol β–DNA complex to accommodate a gapped DNA duplex substrate when the single-stranded gap is short.

Fourth, the fact that the six-nucleotide primer strand was extended by one nucleotide does not necessarily mean that the ternary complex between pol β, DNA and dd-CTP reflects the arrangement during DNA synthesis. As the synthesis on single-stranded DNA substrates is expected to be distributive [68], and the components of the reaction were preincubated for two hours before crystallization, multiple dissociation and reassociation events undoubtedly occurred before the resulting complex was stabilized under the particular set of crystallization conditions.

Finally, if one assumes that the position and orientation of template–primer binding in the pol β–DNA complex can be directly extrapolated to HIV-1 RT, then there are numerous problems with the resulting model (J Ding, E Arnold, unpublished data). First, there are serious steric conflicts between the template strand of the DNA duplex region and the β-sheet in the HIV-1 RT palm subdomain composed of β-strands β12, β13, and β14. It is not possible to generate analogous contacts between residues of the β-sheet containing the HIV-1 RT catalytic site and the template strand without gross movement of the β12-β13-β14 sheet, which seems unlikely as this region is in a similar position in the three different structures of HIV-1 RT determined to date. Second, very little of the binding cleft of HIV-1 RT that was both predicted and observed to be the site of duplex binding [10••,12••] would be in contact with the DNA if the pol β model is applied to HIV-1 RT. Other than a possible interaction with the tip of the β3–β4 loop of the HIV-1 RT fingers subdomain, the primer strand of the DNA would have essentially no contacts with the enzyme. Third, some of the most highly conserved amino acids found in all RNA-dependent polymerases, such as those in motif B of HIV-1 RT, are not proximal to the template–primer if the position of the DNA is what has been observed for the pol β–DNA complex. In the HIV-1 RT–DNA complex, motif B and other nearby elements of the p66 fingers and palm subdomains form part of the 'template grip' for positioning template–primer substrates [12••]; genetic and biochemical data support this role for these residues [35•,57,58,69].

The possibility that the structure of the ternary complex of pol β with ddCTP and two short oligonucleotides described by Pelletier *et al.* [41••] may be partly controlled by crystal packing forces should therefore be considered. Pelletier *et al.*[41••] used this type of argument to raise questions about the biological relevance of a complex between Klenow fragment and oligonucleotides forming an 11 base pair duplex [5••] and the HIV-1 RT–DNA complex.

In summary, although some questions may remain about the biological relevance of the pol β–DNA complexes, there can be little question that the HIV-1 RT–DNA complex is a biologically relevant structure. Moreover, the similarity of the palm subdomains of HIV-1 RT, Klenow fragment, and T7 RNA polymerase strongly suggests that all three of these polymerases will bind DNA substrates in the same orientation.

Reprise

There are a number of issues in polymerization (for example fidelity [70–74], conformational changes that occur during polymerization [38,39,40•,75], processivity [67,73,74,76], drug inhibition and resistance [34•,77,78]) that are ultimately determined by interactions between the enzymes and their substrates. We do not yet have a sufficient understanding of polymerases to be able to point directly to the structural elements responsible for determining these properties; however, the recent progress in structural and biochemical analyses does hold great promise for the future.

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